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14. ABSTRACT

The induction of polyamine catabolism and its production of H2O2 have been implicated in the response to specific anti-tumor polyamine analogues. This project evaluates the ability of Bis(ethyl)norspermine (BENSpm) to induce the polyamine catabolic enzymes in human breast cancer cell lines and examines the role of their induction in the overall response of breast cancer cell lines to BENSpm treatment. To examine the role of the catabolic enzymes in the response of breast cancer cells to the polyamine analogue N1, N11-bis(ethyl)norspermine (BENSpm), a stable knockdown siRNA strategy was used. BENSpm differentially induced SSAT and SMO(PAOh1) mRNA and activity in several breast cancer cell lines, while no N1-acetylpolyamine oxidase PAO mRNA or activity was detected. BENSpm treatment inhibited cell growth, decreased intracellular polyamine levels, and decreased ODC activity in all cell lines examined. The stable knockdown of either SSAT or SMO(PAOh1) reduced the sensitivity of MDA-MB-231 cells to BENSpm while double knockdown MDA-MB-231 cells were almost entirely resistant to the growth inhibitory effects of the analogue. Further, the H2O2 produced through BENSpm-induced polyamine catabolism was found to be derived exclusively from SMO(PAOh1) activity and not through PAO activity on acetylated polyamines. These data suggest that SSAT and SMO(PAOh1) activities are the major mediators of the cellular response of breast tumor cells to BENSpm and that PAO plays little or no role in this response.

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INTRODUCTION

The natural polyamines, spermine, spermidine, and their diamine precursor, putrescine, are ubiquitous polycationic bases that are required for normal cell growth and differentiation (1-4). Breast cancer tissues demonstrate aberrant regulation of the polyamine metabolic pathway, including increased intracellular polyamine levels and decreased activity of SMO(PAOh1), one of the key regulators of polyamine degradation (5, 6). SMO(PAOh1) oxidizes spermine to spermidine and results in the production of H_2O_2, a reactive oxygen species (7-9). Diminished SMO(PAOh1) activity in breast cancer may be associated with reduced levels of H_2O_2 and decreased cell growth inhibition and apoptosis, thereby contributing to breast cancer cell growth. Recently, the activity of SMO(PAOh1) was found to be differentially inducible in several human lung cancer cell lines following exposure to polyamine analogues (10, 11). The hypothesis underlying this project is that induction of SMO(PAOh1) activity by polyamine analogues will deplete intracellular polyamine levels, enhance H_2O_2 production, and lead to growth inhibition and/or activate apoptotic cell death in human breast cancer cell lines.

BODY

Specific Aim 1: To determine the levels of SMO(PAOh1) mRNA, protein expression, and activity in normal and malignant breast cell lines. The induction of SMO(PAOh1) mRNA with BENSpm was first examined by RT-PCR in seven breast cancer cell lines that represent a wide range of breast cancer phenotypes, as well as two immortalized, non-tumorigenic mammary epithelial cell lines. SMO(PAOh1) mRNA was detected at a very low level in each untreated cell line. After treatment with 10 µM BENSpm for 24 hours, a large induction of SMO(PAOh1) mRNA was observed in the two estrogen receptor (ER) negative cell lines, MDA-MB-231 and Hs578t cells, whereas no induction of SMO(PAOh1) mRNA was seen in the two ER positive cell lines, MCF-7 and T47D.

To further investigate the differential induction of SMO(PAOh1) in response to BENSpm treatment, MDA-MB-231 and MCF-7 cell lines, which exhibit a large and small induction of SMO(PAOh1) mRNA, respectively, were chosen for further study. Both cell lines were treated for 24 hours with a range (0 – 50 µM) of BENSpm concentrations. Treatment of MDA-MB-231 cells with BENSpm resulted in a dose-dependent increase in SMO(PAOh1) mRNA expression and enzyme activity whereas no significant change in either parameter was observed using up to 50 µM BENSpm in MCF-7 cells. SMO(PAOh1) mRNA was induced in MDA-MB-231 cells with concentrations up to 10 µM BENSpm, which is the concentration that maximal activity occurred with. This concentration was used to examine the time course of SMO(PAOh1) mRNA and activity induction in both cell lines with BENSpm (0 – 48 hours). Induction of SMO(PAOh1) mRNA in MDA-MB-231 cells began after six hours of treatment with BENSpm with maximal induction achieved by 24 hours. SMO(PAOh1) activity was induced after nine hours and increased through 48 hours of BENSpm treatment. In contrast, SMO(PAOh1) mRNA is minimally induced in BENSpm treated MCF-7 cells after 48 hours and no significant induction is seen in enzyme activity. RT-PCR and real time PCR studies

demonstrated that BENSpm induced the expression of each of the four previously described SMO(PAOh1) variants in MDA-MB-231 cells by about six-fold.

Specific Aim 2: To determine the effects of multiple classes of polyamine analogues on cell growth and death, induction of SMO(PAOh1), related polyamine pathway enzyme activities, and intracellular polyamine levels The induction of SSAT, PAO, and SMO(PAOh1) mRNA and activity by BENSpm was examined in four breast cancer cell lines that represent a wide range of breast cancer phenotypes, MDA-MB-231, Hs578t, MCF-7, and T47D cells using real-time PCR and standard enzyme activity assays. Following treatment with 10 μM BENSpm for 24 hours, SSAT mRNA was induced in all four cell lines following BENSpm exposure while induction of SMO(PAOh1) mRNA was only seen in MDA-MB-231 and Hs578t cells; no PAO mRNA induction was detected in any cell line (Figure 1A). SSAT enzyme activity was induced in each cell line with the greatest induction observed in BENSpm-treated MDA-MB-231 and Hs578t cells (Figure 1B). The induction of SMO(PAOh1) enzyme activity closely correlated with the induction of mRNA with induction only seen in MDA-MB-231 and Hs578t cells (Figure 1C) while no induction of PAO enzyme activity was detected in any of the breast cancer cell lines examined (Figure 1D).

The effects of BENSpm on breast cancer cell growth was examined by treating MDA-MB-231, Hs578t, MCF-7, and T47D cells with 10 μM BENSpm for 96 hours. Treatment of each cell line with 10 μM BENSpm for ≥48 hours significantly inhibited cell growth (Figure 2). Cell growth in each cell line was inhibited similarly by BENSpm through 96 hours of exposure. FACS analysis showed no difference in cell cycle staging in BENSpm-treated MDA-MB-231 and MCF-7 cells; both cell lines arrested in G1 after 48 hours of BENSpm treatment and remained in a G1 block through 96 hours. All four breast cancer cell lines treated with 10 μM BENSpm for 24 hours exhibited a similar decrease of approximately 50% in the levels of spermine, spermidine, and putrescine upon BENSpm treatment with a similar level of BENSpm accumulation in each cell line (Table 1). BENSpm treatment also reduced ODC enzyme activity between 5- and 16-fold in each cell line (Table 1).

To access the relative role played by SSAT and SMO(PAOh1) in determining BENSpm response, RNA interference was used to knock down the expression of each enzyme, alone and in combination, in MDA-MB-231 and MCF-7 cells. These cell lines were chosen as they are representative of both hormone-insensitive and hormone-sensitive breast cancers, respectively, and display different enzyme responses to BENSpm. All data presented here are averages of multiple, independent experiments performed using three clones for each cell type. The knockdown of SSAT and SMO(PAOh1) mRNA and enzyme activity in MDA-MB-231 and MCF-7 cells was confirmed by real-time PCR and enzyme activity assays (Figure 3). In the absence of drug treatment, the growth of these cell lines was not altered by the knockdown of either enzyme alone or in combination.

Intracellular and extracellular polyamines were then measured to examine the effects of the knockdown of SSAT and SMO(PAOh1) on the polyamine content within each cell line. Knocking down SSAT, SMO(PAOh1), or the combination did not significantly

alter the intracellular polyamine levels in untreated MDA-MB-231 or MCF-7 cells, suggesting that basal polyamine homeostasis in untreated breast cancer cell lines is not dependent on either SSAT or SMO(PAOh1) activity (Table 2). BENSpm treatment of MDA-MB-231 ΔSMO(PAOh1) and MCF-7 ΔSMO(PAOh1) cells reduced the intracellular polyamine levels to a similar extent (~70-90%) as in BENSpm-treated vector control cells or BENSpm-treated wild-type parental cell lines (Table 1). However, the knockdown of SSAT, either alone or in combination with SMO(PAOh1), in both MDA-MB-231 and MCF-7 cells reduced BENSpm-induced polyamine depletion such that spermine and spermidine levels were only lowered by ~50% (Table 2). Furthermore, while both acetylspermine and acetylspermidine were detected intracellularly in BENSpm-treated vector and ΔSMO(PAOh1) cells, neither acetylated polyamine was detected in BENSpm-treated ΔSSAT or ΔSSAT/ΔSMO(PAOh1) cells. In addition, acetylspermine was detected in the media in BENSpm-treated vector and ΔSMO(PAOh1) cells but not in the media from BENSpm treated ΔSSAT and ΔSSAT/ΔSMO(PAOh1) MDA-MB-231 cells (data not shown). BENSpm accumulation was similar in each of the cell lines examined. To determine whether the knockdown of SMO(PAOh1) or SSAT, either alone or in combination, affected other parts of the metabolic pathway, effects on ODC, one of the rate-limiting steps in polyamine biosynthesis, were examined. The basal activity level of ODC was similar among all MDA-MB-231 and MCF-7 transfected cell lines (data not shown). ODC enzyme activity significantly decreased in all cell types regardless of SSAT or SMO(PAOh1) knockdown (ODC enzyme activity decreased significantly from 574.1 to 41.7 pmol CO_2/mg prot/hr in BENSpm-treated MDA-MB-231 vector transfected cells and from 520.4 to 25.4 pmol/CO_2/mg prot/hr in BENSpm-treated MDA-MB-231 ΔSSAT/ ΔSMO(PAOh1) cells [$p < 0.001$]; ODC enzyme activity decreased significantly from 1194.8 to 75.2 pmol CO_2/mg prot/hr in BENSpm-treated MCF-7 vector transfeted cells and from 1221.8 to 61.8 pmol CO_2/mg prot/hr in BENSpm-treated MCF-7 ΔSSAT/ΔSMO(PAOh1) cells [$p < 0.001$]).

MTT assays were used to examine the effects of blocking SSAT and/or SMO(PAOh1) induction on the response of MDA-MB-231 and MCF-7 cells to BENSpm treatment (Figure 4). The knockdown of SMO(PAOh1) significantly reduced the sensitivity of MDA-MB-231 cells to BENSpm with concentrations greater than 5 μM BENSpm, but had no effect on the response of MCF-7 cells to BENSpm. Co-treatment of MDA-MB-231 ΔSMO(PAOh1) cells with BENSpm and MDL 72527, the polyamine oxidase inhibitor, did not further alter their sensitivity to BENSpm, providing further evidence that the induction of PAO does not play a role in their response to BENSpm (data not shown). Knockdown of SSAT alone significantly reduced the sensitivity of MDA-MB-231 cells to BENSpm with concentrations greater than 1 μM and modestly reduced the sensitivity of MCF-7 cells to BENSpm (Figure 4). However, MDA-MB-231 ΔSSAT/ΔSMO(PAOh1) cells were significantly less sensitive to BENSpm than either of the single knockdown cells. As expected, the combined knockdown of these two enzymes in MCF-7 cells did not change their response to BENSpm as compared to the knockdown of SSAT alone. Standard cell growth assays were performed to confirm these results using 1 and 10 μM BENSpm treatments for 96 hours in each cell line and similar results were obtained (data not shown). Flow cytometry analysis demonstrated that the knockdown of either SSAT or SMO(PAOh1) in MDA-MB-231 cells reduced the

accumulation of cells in G1 after BENSpm treatment while the knockdown of both enzymes nearly prevented the BENSpm-induced G1 block (data not shown). In MCF-7 cells, the knockdown of SSAT alone reduced the accumulation of cells in G1 whereas dual knockdown did not further alter their response to BENSpm (data not shown).

To determine whether the production of hydrogen peroxide by polyamine catabolism plays a role in the antiproliferative effects of BENSpm and to determine which catabolic pathway is responsible for any H_2O_2 production, CM-H_2DCFDA, an oxidation-sensitive fluorescent probe, was used to detect H_2O_2 production in BENSpm-treated MDA-MB-231 and MCF-7 cells. Treatment of MDA-MB-231 vector transfected cells for 24 hours with 10 µM BENSpm produced a significant increase in fluorescence comparable to that seen for treated wild-type cells (Figure 5). However, co-treatment with either MDL 72527, the polyamine oxidase inhibitor that inhibits both SMO(PAOh1) and PAO enzyme activity, or catalase, which catalyzes the breakdown of H_2O_2, prevented the increase in fluorescence over control (untreated) cells (data not shown). It is important to note that 25 µM MDL 72527 was used to inhibit all oxidase activity in this study, significantly less than 250 µM, which was previously reported in other cell lines (22). To test the possibility that PAO was producing H_2O_2 but the H_2O_2 was rapidly detoxified by peroxisomal catalase, an inhibitor of catalase (AT) was used. Cotreatment of MDA-MB-231 cells with BENSpm and AT, or with BENSpm, AT, and catalase, still resulted in increased fluorescence, while cotreatment with BENSpm, AT, and MDL 72527 did not increase fluorescence (data not shown). Further, no change in fluorescence was seen in MDA-MB-231 ΔSMO(PAOh1) or MDA-MB-231 ΔSSAT/ΔSMO(PAOh1) cells upon any treatment schedule. Exposure of MDA-MB-231 ΔSSAT cells to BENSpm increased fluorescence similar to BENSpm-treated MDA-MB-231 vector transfected cells, indicating that SSAT induction by BENSpm does not lead to the production of H_2O_2 through PAO activity; rather the generation of H_2O_2 in BENSpm-treated MDA-MB-231 cells results primarily from the induction of SMO(PAOh1) activity. All of the controls examined were consistent with SMO(PAOh1) activity being the source of H_2O_2. No evidence of H_2O_2 production was seen in MCF-7 wild-type, vector, ΔSSAT, ΔSMO(PAOh1), or ΔSSAT/ΔSMO(PAOh1) cells with BENSpm (data not shown).

The effects of other classes of polyamine analogues on these parameters will be evaluated in future experiments.

Specific Aim 3: To determine the effects of SMO(PAOh1) over-expression on cell growth and death, related polyamine pathway enzyme activities, and intracellular polyamine levels. Future experiments completed by the pre-doctoral student receiving the remainder of this award will address this aim.

KEY RESEARCH ACCOMPLISHMENTS

- BENSpm differentially induces SMO(PAOh1) mRNA and activity in multiple human breast cancer cell lines.
- BENSpm induces SMO(PAOh1) mRNA and activity in a time- and dose-dependent manner in MDA-MB-231 cells.
- BENSpm induces the mRNA expression of each of the four identified SMO(PAOh1) splice variants 4-6 fold in MDA-MB-231 cells.
- BENSpm exposure inhibits cell growth, reduces intracellular polyamine levels, induces SSAT activity, and reduces ODC activity in multiple breast cancer cell lines regardless of SMO(PAOh1) induction.
- No induction of PAO mRNA or activity was seen in any of the BENSpm-treated breast cancer cell lines examined.
- BENSpm-induced oxidase activity utilized only spermine, the preferred SMO(PAOh1) substrate, as a substrate in MDA-MB-231 and Hs578t cells and showed no activity with either N^1-acetylspermine, the preferred PAO substrate, or spermidine.
- Co-treatment of MDA-MB-231 and Hs578t cells with BENSpm and MDL 72527, the polyamine oxidase inhibitor, significantly reduces their sensitivity to BENSpm but does not alter the sensitivity of MCF-7 and T47D cells to BENSpm.
- In MDA-MB-231 cells, blocking the induction of either SMO(PAOh1) or SSAT individually using stable siRNAs reduces their sensitivity to BENSpm.
- When the induction of both SSAT and SMO(PAOh1) is prevented, MDA-MB-231 cells become significantly more resistant to the growth inhibitory effects of BENSpm than the vector controls or the individual knockdowns alone, demonstrating that the antiproliferative effects of BENSpm can be mediated through both SSAT and SMO(PAOh1) induction.
- Hydrogen peroxide produced in BENSpm exposed MDA-MB-231 cells originates from SMO(PAOh1) activity induction, not from PAO activity as previously proposed.
- BENSpm-induced SSAT enzyme activity results in the acetylation of spermine and spermidine, which are subsequently exported from the cell rather than serving as substrates for PAO.

REPORTABLE OUTCOMES

- ***Abstracts:***
  - **Differential induction of human spermine oxidase mRNA and activity in human breast cancer cell lines.** Pledgie A, Huang Y, Hacker A, Woster PM, Casero RA, and Davidson NE. Proc AACR. Page 1224, 2004
- ***Publications:***
  - **Spermine oxidase SMO(PAOh1), not N1-acetylpolyamine oxidase PAO, is the primary source of cytotoxic H2O2 in polyamine analogue-treated human breast cancer cell lines.** Pledgie A, Huang Y, Hacker A, Zhang Z, Woster PM, Davidson NE, and Casero RA Jr. J Biol Chem, 280: 39843-41, 2005.

- ***Presentations:***
  - **The role of spermine oxidase in human breast cancer.** Pledgie A. Breast Cancer Program Seminar Series, Department of Oncology, Johns Hopkins University, May 2004
- ***Doctor of Philosophy***
  - **Biochemistry and Molecular Biology,** Johns Hopkins Bloomberg School of Public Health, May 2006

CONCLUSIONS

This study demonstrates that SSAT and SMO(PAOh1) are major targets of BENSpm in certain human breast cancer cell lines and, further, that these catabolic enzymes act together to determine cellular response to BENSpm. The antiproliferative effects of BENSpm in MDA-MB-231 cells are mediated, in part, through the production of H_2O_2 by SMO(PAOh1) and by the export of acetylated polyamines formed by the activity of SSAT. Future studies should be directed to explore the ability of additional polyamine analogues to induce SMO(PAOh1) and SSAT activity; such induction will lead to the production of H_2O_2 that may result in cell growth inhibition and/or apoptotic cell death. Another noteworthy finding was the negative correlation between the induction of the polyamine catabolic enzymes SMO(PAOh1) and SSAT and the expression of the estrogen receptor-alpha (ER). These results suggest a potential negative role of ER in the expression of these catabolic enzymes; further understanding of the transcriptional regulation of SMO(PAOh1) and SSAT will aid in the development of more cytotoxic agents to use against breast cancer. In conclusion, the results from this study demonstrate the independent effects of the polyamine catabolic enzymes in response to polyamine analogue treatment and provide insight for the development of more specific anticancer agents for the treatment of human breast cancer.

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SUPPORTING DATA

Table 1. Effects of BENSpm treatment on intracellular polyamine levels and ODC activity. MDA-MB-231, Hs578t, MCF-7 and T47D cells were treated with 10 μM BENSpm for 24 hours. Intracellular polyamine levels and ODC enzyme activity were assayed as described in Materials and Methods. Values are the means ± SD of three independent experiments in duplicate for the polyamines and three independent experiments in triplicate for ODC enzyme activity.

		Polyamines (nmol/mg protein)			BENSpm	ODC Activity
Cell line	Treatment	Putrescine	Spermidine	Spermine	(nmol/mg protein)	(pmol CO_2/mg protein/h)
MDA-MB-231	Control	3.9 ± 0.2	36.9 ± 4.6	47.0 ± 8.2	ND	577.9 ± 26.1
	BENSpm	2.3 ± 0.3	10.9 ± 1.2	20.6 ± 3.4	38.5 ± 4.4	76.7 ± 4.8
Hs578t	Control	2.6 ± 0.7	46.6 ± 3.2	45.3 ± 2.9	ND	480.6 ± 19.5
	BENSpm	1.2 ± 0.2	15.7 ± 1.6	22.2 ± 1.1	45.2 ± 4.7	93.4 ± 9.2
MCF-7	Control	6.2 ± 0.3	65.8 ± 6.9	52.2 ± 7.8	ND	1525.7 ± 116.6
	BENSpm	2.7 ± 0.2	26.8 ± 5.4	26.4 ± 4.7	52.3 ± 5.9	93.4 ± 12.4
T47D	Control	4.3 ± 0.5	60.7 ± 4.4	47.0 ± 2.8	ND	1264.6 ± 65.3
	BENSpm	2.2 ± 0.4	26.2 ± 1.7	14.7 ± 0.9	42.6 ± 2.4	103.1 ± 6.5

Table 2. Effects of BENSpm treatment on intracellular and extracellular polyamine levels. Cells were treated with 10 μM BENSpm for 24 hours. Intracellular polyamine levels in A) MDA-MB-231 and B) MCF-7 cells were assayed as described in Materials and Methods. Values are the means ± SD of three independent experiments performed in duplicate. ND= not detected.

A.

	Polyamines (nmol/mg protein)					BENSpm
Cell line and Treatment	Putrescine	Spermidine	Spermine	Acetylspermine	Acetylspermidine	(nmol/mg protein)
231 vector control	2.3 ± 0.2	34.1 ± 1.2	48.9 ± 2.5	ND	ND	ND
231 vector BENSpm	ND	8.7 ± 0.7	12.1 ± 1.1	0.7 ± 0.1	4.4 ± 0.7	38.6 ± 3.7
231 ΔSMO(PAOh1) control	2.8 ± 0.3	32.5 ± 3.4	54.3 ± 2.4	ND	ND	ND
231 ΔSMO(PAOh1) BENSpm	ND	8.9 ± 0.9	13.9 ± 2.9	1.9 ± 0.3	8.8 ± 0.5	36.4 ± 2.1
231 ΔSSAT control	1.8 ± 0.3	38.6 ± 1.4	39.8 ± 2.5	ND	ND	ND
231 ΔSSAT BENSpm	ND	20.6 ± 0.5	19.1 ± 0.8	ND	ND	41.3 ± 4.9
231 ΔSMO(PAOh1)/ΔSSAT control	1.9 ± 0.2	35.7 ± 1.5	41.1 ± 1.8	ND	ND	ND
231 ΔSMO(PAOh1)/ΔSSAT BENSpm	ND	20.1 ± 0.4	26.4 ± 0.5	ND	ND	38.5 ± 3.1

B.

	Polyamines (nmol/mg protein)					BENSpm
Cell line and Treatment	Putrescine	Spermidine	Spermine	Acetylspermine	Acetylspermidine	(nmol/mg protein)
MCF-7 vector control	3.7 ± 0.1	68.3 ± 5.6	50.3 ± 4.4	ND	ND	ND
MCF-7 vector BENSpm	1.3 ± 0.2	7.5 ± 0.6	5.1 ± 0.5	8.7 ± 0.6	15.9 ± 0.7	47.1 ± 2.5
MCF-7 ΔSMO(PAOh1) control	3.8 ± 1.0	60.1 ± 6.6	59.2 ± 5.6	ND	ND	ND
MCF-7 ΔSMO(PAOh1) BENSpm	0.9 ± 0.2	7.9 ± 1.1	6.7 ± 0.6	7.9 ± 0.4	11.2 ± 0.6	42.8 ± 3.2
MCF-7 ΔSSAT control	3.1 ± 0.6	65.1 ± 6.9	57.1 ± 3.9	ND	ND	ND
MCF-7 ΔSSAT BENSpm	1.9 ± 0.1	52.4 ± 5.2	40.9 ± 2.5	ND	ND	46.2 ± 1.8
MCF-7 ΔSMO(PAOh1)/ΔSSAT control	2.9 ± 0.3	64.7 ± 4.8	59.7 ± 6.3	ND	ND	ND
MCF-7 ΔSMO(PAOh1)/ΔSSAT BENSpm	1.0 ± 0.3	51.3 ± 4.1	44.5 ± 2.7	ND	ND	43.9 ± 1.9

Figure 1. BENSpm induces SSAT and SMO(PAOh1) mRNA and activity in multiple human breast cancer cell lines. MDA-MB-231, Hs578t, MCF-7, and T47D breast cancer cell lines were treated with 10 µM BENSpm for 24 hours. A) Real-time PCR for SSAT mRNA (black bars), PAO mRNA (white bars), and SMO(PAOh1) mRNA (gray bars) was performed as described in Materials and Methods; all values were normalized to the GAPDH housekeeping gene. Values are the means ± SD of four independent experiments performed in duplicate. B) SSAT activity was determined as described in Materials and Methods. C) SMO(PAOh1) activity was assayed as described in Materials and Methods using 250 µM spermine as the substrate. D) PAO activity was determined as described in Materials and Methods using 250 µM N1-acetylspermine as the substrate. Enzyme activity values are the means ± SD of three independent experiments performed in triplicate.

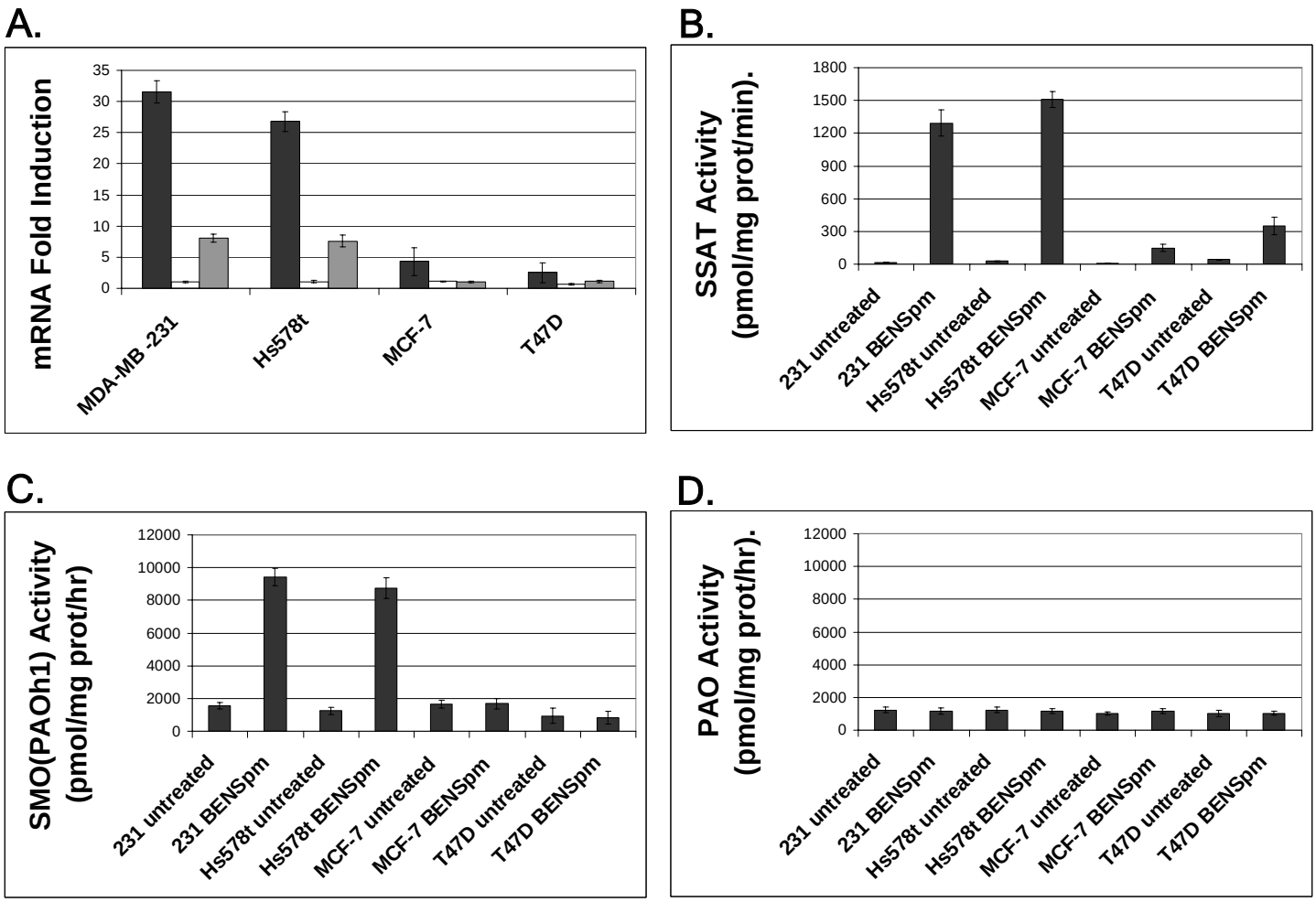


Figure 2. BENSpm inhibits the growth of multiple breast cancer cell lines. MDA-MB-231, Hs578t, MCF-7, and T47D breast cancer cell lines were treated with 10 μM BENSpm for 96 hours. Cells were detached with trypsinization and were counted every 24 hours as described in Materials and Methods. The results are the means ± SD of three independent experiments performed in triplicate with $p<0.001$ after 48 hours of BENSpm treatment in each cell line as determined using a mixed-effects model.

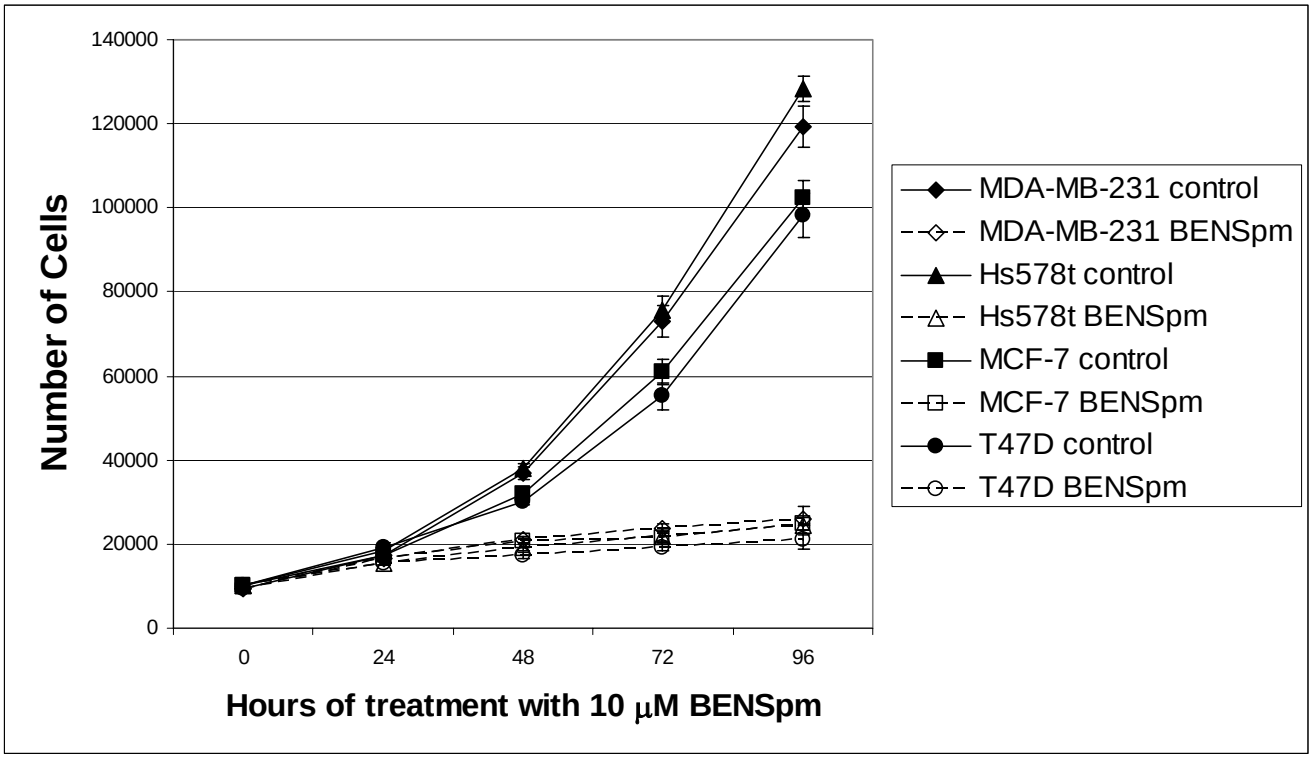
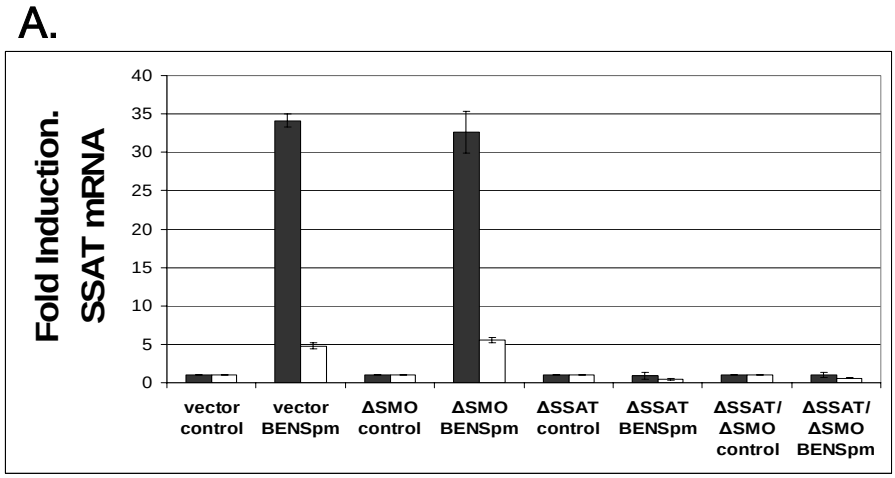
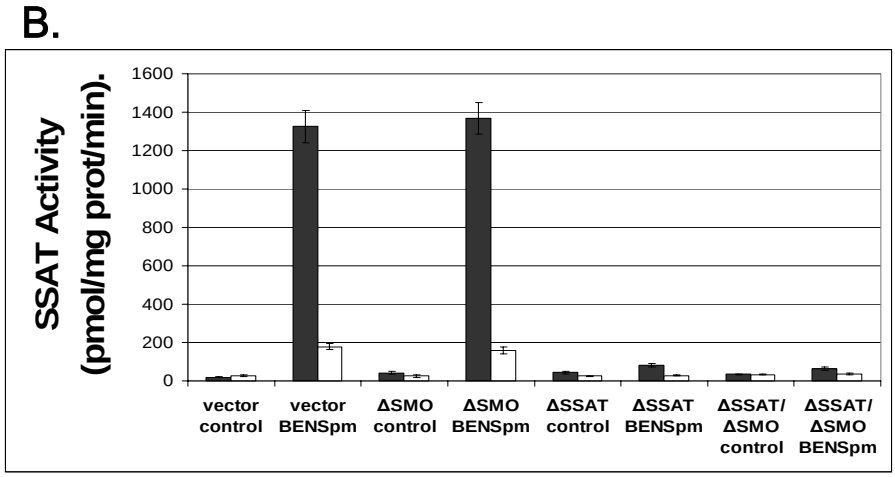
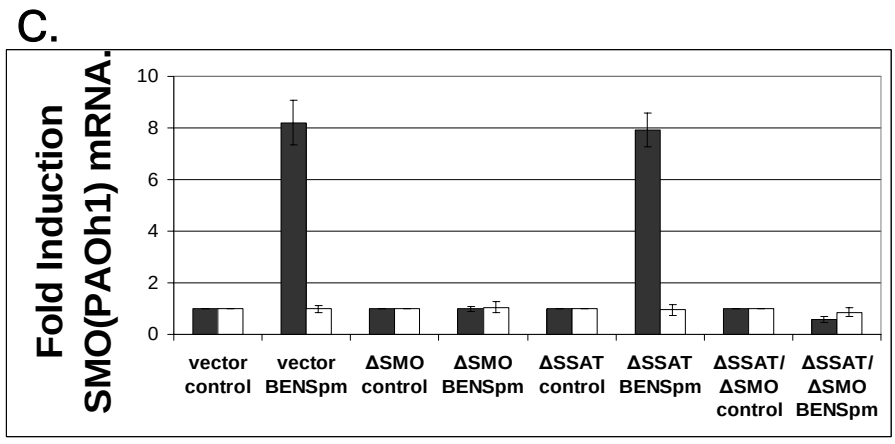


Figure 3. siRNA directed against SMO(PAOh1) and SSAT specifically and efficiently reduces respective SSAT and SMO(PAOh1) mRNA and activity induction by BENSpm. Transfected MDA-MB-231 cells (black bars) and MCF-7 cells (white bars) were treated with 10µM BENSpm for 24 hours. A) Real-time PCR for SSAT mRNA and C) real-time PCR for SMO(PAOh1) mRNA was performed as described in Materials and Methods; all values were normalized to the GAPDH housekeeping gene. Values are the means ± SD of four independent experiments performed in duplicate. B) SSAT enzyme activity was assayed as described in Materials and Methods. D) SMO(PAOh1) enzyme activity was assayed as described in Materials and Methods using 250 µM spermine as the substrate. Enzyme activity values are the means ± SD of three independent experiments performed in triplicate.







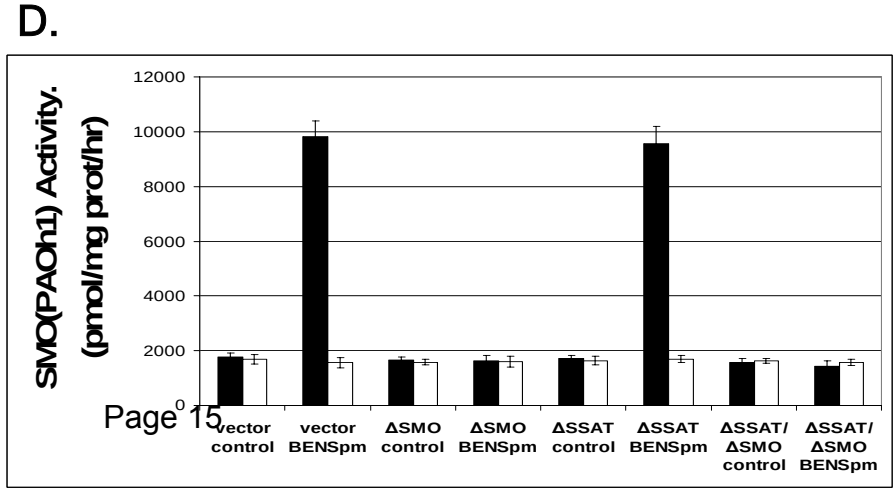


Figure 4. Effects of SMO(PAOh1) and SSAT knockdown on the sensitivity of breast cancer cell lines to BENSpm. Transfected MDA-MB-231 (A) and transfected MCF-7 (B) cells were exposed to increasing concentrations (0.1 to 25 μM) of BENSpm for 96 hours. The effect on cell growth was assayed using the MTT assay as described in Materials and Methods. The results are the means ± SD of three independent experiments performed in quadruplicate. ANCOVA analysis demonstrated that for BENSpm ≥5 μM, MDA-MB-231 ΔSMO(PAOh1) and MDA-MB-231 ΔSSAT cells were statistically less sensitive to BENSpm than MDA-MB-231 vector cells **(**p=0.020 and p=0.005, respectively**)**. MDA-MB-231 ΔSSAT/ΔSMO(PAOh1) cells were statistically less sensitive to BENSpm than either of the single knockdowns (p<0.001 and p<0.001). There was no statistically significant difference in the growth of cells between any of the MCF-7 cell lines.

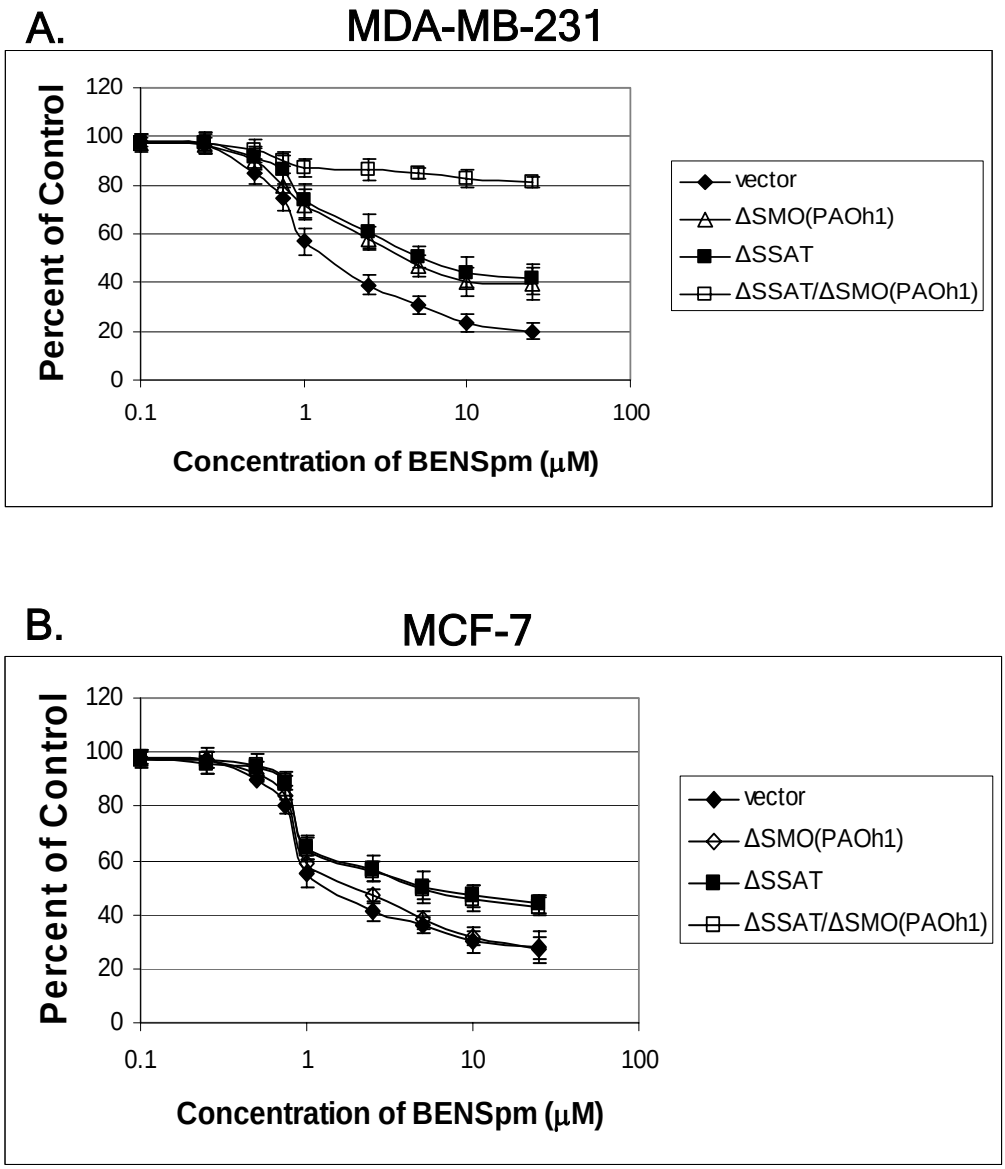


Figure 5. Effects of BENSpm-induced fluorescence in MDA-MB-231 cells detected by CM-H2DCFDA. MDA-MB-231 vector-transfected (A), MDA-MB-231 ΔSMO(PAOh1) (B), MDA-MB-231 ΔSSAT, and MDA-MB-231 ΔSMO(PAOh1)/ ΔSSAT cells were treated with 10 μM BENSpm for 24 hours, harvested, and treated with 10 μM CM-H2DCFDA for 30 minutes. $1x10^5$ cells were analyzed by flow cytometry as described in Materials and Methods. The X-axis represents F1 fluorescence intensity and the Y-axis represents cell number. Shown are representative results from one of three experiments that gave similar results.